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Mechanisms and Treatment of Lung Lesions and Associated Surfactant Damage In Shock.

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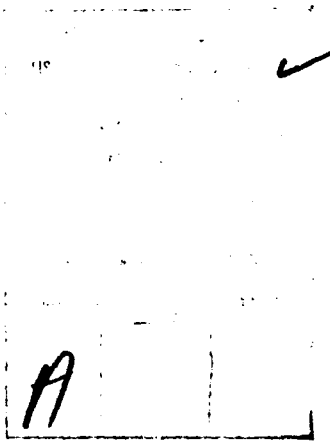

ABSTRACT

A. Development of a rapid and simple method for lung surfactant purification:

A major problem in the study of human lung surfactant, particularly in pathologic conditions, is the lack of appropriate methods to separate the surfactant from blood components transudated into the air spaces. A rapid and simple method capable of purifying surfactant from alveolar washings, from washings contaminated with plasma and from residual lung tissue (intracellular surfactant) has been developed. The sample, containing 16% NaBr, is placed beneath a two-layer discontinuous gradient of NaBr. After centrifugation, the surfactant is found near the top of the gradient tube at a density of 1.085 at 4°C while the contaminating material remains near the bottom. The chemical composition of surfactant from lung washings of normal animals isolated by this method compares quite favorably with surfactant isolated by much more elaborate and time consuming methods. In addition, a surfactant fraction with a chemical composition very similar to that of the surfactant from normal lung washing can readily be separated from a mixture of plasma and lung washings by this method. Application of the method to the lung tissue remaining after thorough alveolar lavage permits recovery of an intracellular surfactant fraction which has a somewhat different composition than alveolar surfactant isolated from lung washings. This method has also been applied to small samples using as little as 1 ml of starting material for the isolation of surfactant, making the method quite useful for isolating surfactant from human tracheal aspirates in order to monitor clinically surfactant changes in patients with adult respiratory distress syndrome.

B. Biochemical and structural changes of the lung surfactant in endotoxic shock:

(1) Biochemical changes: Purification and biochemical evaluation of the alveolar and intracellular surfactant has already been performed in two pairs of monkeys. One monkey from each pair was infused with 10 mg endotoxin/kg/hr while the other monkey received Ringer's lactate solution only. The animals were killed 10 hours after the onset of endotoxin or saline infusion. Biochemical evaluation of the lung washings from the above monkeys revealed the presence of large excesses of protein in the washing from the endotoxin treated animals due to the pulmonary edema. However, following density gradient centrifugation of the experimental and control washings, contaminating plasma lipids and proteins were eliminated and the lecithin composition of both control and experimental monkeys were similar with the exception that one of the endotoxin injected monkeys (which also revealed prominent pulmonary edema) the percent of the palmitic acid of surfactant lecithin was lower than normal. However, evaluation of the intracellular surfactant failed to demonstrate differences in the biochemical composition of surfactant between control and experimental groups. The above preliminary studies suggest that the early development of "congestive atelectasis" in shock is not associated with apparent profound changes in the surfactant system.

(2) Structural changes: Using the method of total lung fixation (which was found to preserve the surfactant layer and the overall lung architecture) we ~~were able to~~ demonstrated that congestive atelectasis is characterized by multifocal foldings of the alveolar walls which characteristically involve damaged areas of the (injury sensitive) type I epithelium. The multifocal foldings resulted in the formation of small plicated alveoli. The surfactant layer was found to be intact only in non-plicated portions of the alveolar wall and revealed excess focal deposition of myelin figures (surfactant phospho-

lipids) sealing the openings of the folded portions of the alveolar epithelial lining. These findings suggest that the surfactant layer cannot be readily formed at sites of epithelial injury and, therefore, the injured parts of the epithelial lining plicates during expiration.

C. Coagulation and other hematologic changes in endotoxin shock with or without steroid treatment were recorded and

Initially we investigated the hematologic changes induced by continuous endotoxin infusion (10/mg/kg/hr) versus bolus endotoxin infusion (10 mg/kg injected over a 10 min. period). A major difference between the two models was the finding that in the bolus model factor XII dropped sharply within 30 min. after endotoxin infusion. By contrast in the continuous infusion model the level of factor XII actually increased after 30 min. but thereafter demonstrated a gradual but progressive drop. All other hematologic and coagulation parameters were essentially the same in both models during the first three hour period after the onset of endotoxoin injection. In the continuous infusion model with or without steroid treatment the following hematologic changes were observed: (1) Leukocytes: marked drop during the first three hours followed by rebound progressive increase thereafter. At 10 hours the leukocyte count had reached the pre-infusion level and at 16-18 hours the animals showed slight leukocytosis. By contrast steroid treated animals showed only mild drop in the leukocyte count at 30 min. Thereafter there was a sharp increase and at 6 hours the leukocyte count ($1000/mm^3$) was 22.5 ± 5.6 (baseline value 10.3 ± 1.8). In addition further progressive increase in the leukocytes count was noted throughout the 24 hour experimental period. (2) Platelets and fibrinogen: they both demonstrated progressive drop in the continuous infusion model, but this drop was significantly less prominent in animals treated with steroids. (3) Coagulation factors: At 30 min. no significant changes were noted. After 3 hours factors V, VIII, and XII demonstrated progressive decrease while the drop of factor VII was marked only at 16-18 hours. Treatment with glucocorticosteroids significantly inhibited the drop of factor VIII but had no statistically significant affect on factor V and XII.

The above hematologic data correlated well with the histochemical and ultrastructural findings which indicated that glucocorticoids inhibit endotoxin induced : (a) sequestration degranulation and fragmentation of the leukocytes in the pulmonary microcirculation, (b) marked endothelial damage in association with interstitial edema, multifocal swelling of the type I epithelium and congestive atelectasis, and (c) early development of diffuse microthrombosis in the sinusoids of the liver (and spleen) as well as centrilobular liver necrosis.

D. The effect of epsilon aminocaproicacid (EACA) and trasylol on endotoxin shock was noted.

(1) EACA: Four pairs of monkeys were used. In each pair of monkeys endotoxin was infused continuously at a rate of 10 mg/kg/hr but one monkey from each pair also received EACA 50 or 100 mg/kg/hr. The monkeys were killed in pairs at 6 hours and 16 hours after the onset of endotoxin injection. The results that we have obtained thus far suggest that this drug does not significantly alter endotoxin induced hematologic changes but does appear to inhibit margination

of the leukocytes in the pulmonary vascular bed during the early stages of shock. These changes were associated with significant preservation of the endothelial integrity. In addition, there was evidence that EACA inhibited formation of fibrinous deposits in the microcirculation of the liver and spleen possible because the drug promoted endothelial preservation. In future experiments EACA will be given in smaller doses, 20 mg/kg/hr, because a significant transient hypotensive response was noted rapidly after the onset of EACA administration and this response was more severe with 100 mg EACA/kg/hr. (2) Trasylol: Four pairs of monkeys were infused with endotoxin (10 mg/kg/hr) but one animal from each pair was also given trasylol, 5,000 KIU/kg/hr. The animals were sacrificed 6 or 10 hours after the onset of endotoxin infusion. We have not as yet completed evaluation of the above pilot experiments because we are currently concentrating on the in vitro affects of trasylol on blood coagulation. However, our preliminary findings suggest that the doses employed were large since one animal developed disseminated intravascular thrombosis.

MECHANISMS AND TREATMENT OF LUNG LESIONS AND ASSOCIATED SURFACTANT DAMAGE IN SHOCK

Development of a rapid and simple method for lung surfactant purification. (See also appended manuscript). The results of the study designed to investgate the nature of the lipid-protein complexes of lung washing has led to the development of a simple, rapid method for the purification of lung surfactant. This method avoids sedimentation of the surfactant material and is, therefore, applicable to samples that are contaminated with large quantities of protein, cell debris or other non-surfactant components. Solid NaBr is added to the sample to give a density of 1.14 and the sample is then placed beneath a two-layer discontinuous gradient. Surfactant has a density lower than that of the contaminating materials and moves upward through the gradient during the 3 hr. centrifugation at 4°C and 90,000 g (max.), while other components do not move upward unless present in very large concentrations. In these instances, the surfactant can be purified by removing the surfactant band, and after appropriate density adjustment, placing it beneath another gradient and centrifuging in the same manner. In our earlier studies (1), the sample was layered beneath a continuous density gradient of NaBr; however, since NaBr has a low viscosity and tends to form continuous gradients when centrifuged, the procedure was simplified by use of a discontinuous gradient.

The surfactant isolated from rabbit lung washings by this method contained a high concentration of phospholipid and relatively little protein (average phospholipid to protein ratio was 13 to 1). The surfactant was highly surface active and appeared as small liposomes measuring up to 100 nm in diameter when examined by electron microscopy following negative staining. More than 85% of the phospholipids of the surfactant band were PC*, with phosphatidyl glycerol (PG) being the next most abundant phospholipid and with smaller quantities of phosphatidyl ethanolamine (PE) and phosphatidyl inositol (PI) present. The major fatty acid of PC was palmitic acid (16:0) which accounted for almost 70% of the total PC fatty acids. These findings are consistent with the expected biochemical composition of lung surfactant.

To determine if this simple method of surfactant purification was capable of separating surfactant from plasma proteins and lipids often found in lung washings, especially in various pathological conditions, lung washings and plasma were mixed prior to density gradient centrifugation. A particulate band, rich in phospholipids, was found in the same position as was the surfactant from lung washings only. However, only about 38% of the total phospholipid from the mixture was in the surfactant band compared with about 80% from lung washings only. Although less than 1% of the total protein in the gradient was present in the surfactant fraction, the very large amount of protein found in plasma resulted in a considerable contamination of the surfactant band with protein. The fact that the density at which the band was located was the same as for washings only indicated that this excess protein was not associated with the surfactant phospholipid. This was also shown by using a part of the surfactant band to prepare a second gradient. After centrifugation of the second gradient, only a small peak of protein remained with the phospholipid band. Eight percent of the protein and 87% of the phospholipid of the gradient were in this band. Purification of the plasma-washing mixture on density gradients removed plasma lipids as well as protein. The plasma-washing mixture contained more lysophosphatidylcholine (LPC), sphingomyelin (Sph) and PE and less PC and PG than the lung washings. The surfactant from both the first and

*PC, phosphatidyl choline

second gradients contained less of the plasma components, LPC, Sph and PE, and more of the surfactant phospholipids, PC and PG, with the final surfactant band having a phospholipid composition similar to that of the surfactant from the lung washings only. The same pattern of changes could be seen in the fatty acid composition of the PC, with the plasma-washing mixture containing less palmitic and more stearic, linolenic and arachidonic acids than the lung washings. Gradient centrifugation resulted in the purification of a surfactant band which contained more palmitic and less stearic, linolenic and arachidonic acids than the original mixture. Although not identical, this fatty acid distribution closely resembled that of surfactant from lung washings.

This method of density gradient centrifugation was also applied to lung tissue remaining after alveolar lavage (residual lung tissue) in an attempt to isolate intracellular surfactant. Following vigorous homogenation of the tissue, intended to permit complete recovery of the intracellular surfactant and not expected to yield intact lamellar bodies, and a low speed centrifugation (to remove connective tissue and cell debris), the lung homogenate was used to prepare gradients in the same way as lung washing. Following centrifugation, a band of particulate material was clearly visible at the same position in the tube as for lung washing surfactant. About 40% of the phospholipid of the lung homogenate was found in the band along with less than 1% of the protein. The surfactant fractions from the residual lung tissue were surface active, but did not reduce the minimum surface tension to the very low values found with lung washing surfactant. Electron microscopic examination of the phospholipid containing band from residual lung tissue showed small liposomes in the negatively stained preparation similar to those in lung washing surfactant. However, thin sections revealed that this fraction from residual lung tissue contained occasional electron lucent or moderately electron dense droplets which were interpreted to be neutral lipids. This interpretation was supported by thin layer chromatography findings. Residual lung surfactant contained considerably more cholesterol, free fatty acids and triglycerides than did the surfactant isolated from lung washings, which may account for reduced ability of the intracellular surfactant to lower surface tension. Phospholipid analysis of the residual lung homogenate and the surfactant purified from it revealed that the amount of PC increased after density gradient centrifugation from 50% to 70% and the palmitic acid content of the PC increased from 43% to 57%. However, the residual lung surfactant purified in this manner has a phospholipid composition somewhat different from that of the surfactant from lung washings.

This simple and rapid method of density gradient centrifugation had been shown to be capable of separating surfactant from large amounts of contaminating protein and non-surfactant lipids as demonstrated by the recovery of a surfactant band from gradients prepared with plasma-lung washing mixture or lung homogenates. The chemical composition of the surface-active fractions obtained by this method compares quite favorably with surfactant isolated by much more elaborate and time consuming methods, which are not applicable to small samples or to samples contaminated with large quantities of protein. Recently we have adapted this method to a smaller scale for use with tracheal aspirate samples. One ml (instead of 20 ml) of sample is placed beneath a 4 ml (instead of 40 ml) gradient and ecntrifuged for one hour.

The above method will be extensively used in the future, not only in animal and human lungs but also in human tracheal aspirates to monitor clinically the

state of surfactant in various forms of adult respiratory distress syndrome. We have already initiated such studies in tracheal aspirates from newborn infants with respiratory distress as part of a related project, supported by the NIH, which is concerned with the pathophysiology of surfactant during the perinatal period. I am enclosing separately a summary of the progress we have made on pharyngeal and tracheal aspirates from newborns because these results are directly relevant to our future studies on adult tracheal aspirates.

B. Biochemical and Structural Changes of the Lung Surfactant in Endotoxic Shock. Surfactant damage has been related to shock and other pathological conditions associated with pulmonary edema and congestive atelectasis. However, the nature, mechanisms and significance of surfactant changes in the above disease processes are still unknown. It is generally assumed that alteration of the surfactant layer by disease may be the result of deficiency in synthesis and secretion of surfactant components, mechanical disruption of the layer, contamination of the layer by aspiration or inactivation of surfactant following transudation of blood constituents into the air space. In addition, the structural and functional integrity of the surfactant layer requires an intact alveolar epithelial lining and, therefore, alterations of the surfactant layer may occur following damage of the injury sensitive type I alveolar epithelium. It is apparent from the above considerations that significant progress in the field of surfactant pathophysiology will be made only with close correlation of appropriate parameters relating to the biochemical, functional and structural characteristics of normal and altered surfactant system.

The studies described below were initiated following the development of appropriate methods for the isolation and purification of intracellular and extracellular surfactant as well as surfactant contaminated with blood constituents (see Section A, page 4).

1. Biochemical changes of surfactant in endotoxin-induced congestive atelectasis of the lung. We have performed thus far detailed biochemical studies in two pairs of monkeys. One monkey from each pair was infused with 10 mg endotoxin/kg/hr. while the other monkey received Ringer's lactate solution. The animals were killed 10 hours after the onset of endotoxin or saline infusion. Multiple lung washings (a total of 10 changes) were obtained by alveolar lavage from the left lung using a standardized procedure designed to remove completely the extracellular (alveolar) surfactant. These lung washings and the residual tissue were stored at -70°C and evaluated biochemically at a later date. Previous pilot studies have shown that storage of washing and lung specimens at this temperature do not alter the biochemical and functional characteristics of the surfactant system. From the lung washing and residual lung tissues gradients were prepared for isolation and purification of surfactant using methods described in section A and in the appended manuscript. As it was expected from the morphological studies, the lung washing of the endotoxin-treated monkeys contained large amounts of protein due to the associated pulmonary edema. However, following density grading centrifugation most of the contaminating plasma proteins and lipids were eliminated and the surface tension properties of the surfactant fractions from the experimental and control monkeys were essentially identical. The phospholipid changes of the surfactant fractions in one pair of monkeys are summarized in Table Ia. The data suggests that the control and endotoxin treated monkeys do not defer significantly with respect to the amount and composition of surfactant phospholipids. In addition, the fatty acid composition of the intracellular as well as extracellular phosphatidyl

choline was identical in control and test animals. In the second pair of monkeys the biochemical profiles of the surfactant system were essentially the same in both animals with the exception that the percent of the palmitic acid of the extracellular surfactant lecithin was lower in the endotoxin treated animal (65.5%) than in the control (75%). No differences, however, were found in the fatty acid composition of the intracellular surfactant. The above findings, although still preliminary, suggest that the early development of congestive atelectasis in shock is not associated with profound qualitative changes in the surfactant system. These findings, however, do not exclude the possibility that the surfactant is inactivated following transudation of plasma proteins into the air space. Moreover, the above findings provide no information as to whether surfactant release into the air spaces is normal in shock lung. In this respect the amount of intracellular and extracellular surfactant was determined in one of the two pairs of monkeys. Although the total (combined intracellular and extracellular) amount of surfactant is the same in both control and test monkeys (Table Ia), there appears to be an increase of the intracellular and a decrease of the extracellular surfactant PL in the endotoxin treated animal (Table Ib). This finding - if confirmed with additional experiments - would suggest that the rate of surfactant secretion in endotoxic shock is reduced predisposing to atelectasis.

In order to evaluate whether surfactant inactivation does occur following transudation of plasma proteins into the alveoli, binding studies should be performed to investigate whether plasma versus non-plasma proteins associated with the surfactant system interact in a different manner with the surfactant lipids. Such studies are currently in progress. In addition, radioisotope tracing studies will be performed to evaluate whether the rate of synthesis and secretion of surfactant lecithin is altered following prolonged endotoxin shock. At present no data has been reported relevant to the turnover of surfactant components in monkeys, although various radioactively labeled surfactant precursors including radioactive acetate have been extensively used in animals other than primates. In a pilot study, however, we unexpectedly found that radioactive acetate injected intravenously three hours prior to sacrifice of two monkeys did not incorporate into the lipids of lung washings. Moreover, evaluation of the lipids from residual lung homogenates revealed that only cholesterol showed activity. At present we have no explanation for the above unusual findings. In the future we will perform radioisotope tracing studies utilizing ^{14}C - choline which is known to be preferentially incorporated in the surfactant lecithin.

2. Structural changes of the surfactant layer and underlying epithelium in endotoxin-induced congestive atelectasis. One of the methods which has been previously developed for the preservation of the surfactant layer is total fixation of the lung prior to sectioning (2). During the past year we extensively used a modification of this method because we found that it preserves not only the surfactant layer but also the in vivo state of alveolar expansion and overall architecture of the lung. More important the method was found to be superior to study the mechanism of congestive atelectasis. The method is briefly as follows: Following sacrifice of the animal the trachea is ligated in order to prevent collapse of the lung after thoracotomy. Subsequently the major bronchi are ligated and the excised lung lobes with the ligature still attached are fixed in toto for 24 hours. The lung lobes are then sectioned for light and electron microscopy. It should be mentioned that only periph-

eral portions of glutaraldehyde-fixed lung lobes are used for transmission and scanning electron microscopy because fixation is incomplete in more central areas of the lung. Using the above method we were able to demonstrate that congestive atelectasis is characterized by multifocal foldings of the alveolar walls which characteristically involve damaged areas of the (injury sensitive) type I epithelium. The multifocal foldings resulted in the formation of small plicated alveoli. The surfactant layer was found to be intact only in nonplicated portion of the alveolar wall and revealed excess focal deposition of myelin figures (surfactant phospholipids) sealing the openings of the folded portions of the alveolar epithelial lining. These findings suggest that the surfactant layer cannot be readily formed at sites of epithelial injury and, therefore, the injured parts of the epithelial lining plicates during expiration. If this interpretation is correct, one would expect that during inspiration the alveoli unfold and excess transudation of fluid exudes from the damaged portions of previously plicated type I epithelium. We have also observed that the pores of Cohn (by which adjacent alveoli intercommunicate) are frequently occluded due to the swelling of their epithelial lining. The pores of Cohn can be readily seen by scanning electron microscopy and we are currently investigating their distribution in normal alveoli as well as alveoli from shock lungs with or without treatment.

On the basis of the above findings the following questions must be answered: Is the so-called "respirator lung" caused, at least in part, by increased transudation of fluid during the inspiratory phase which results in unfolding of previous damaged portions of the type I epithelium? What is the diameter of the alveoli and of the pores of Chon as well as the thickness of the alveolar septa and surfactant layer during inspiration and expiration in normal versue shock lung? In order to answer the above questions a more sophisticated procedure has been recently devised. This procedure has been already successfully tested in rabbits and is briefly as follows: Following tracheostomy under pentobarbital anesthesia, the animals are intubated and placed on respirator prior to sacrifice. A thoracotomy is then performed, the right middle bronchus is ligated and sections of right middle lobe are rapidly taken for light and electron microscopy. Subsequently, the right and left lower bronchi are doubly ligated sequentially at the end inspiratory phase (20 cm water pressure) and during expiration (at 5 cm water pressure) or at the end expiratory phase, respectively. Following these procedures, both lower lobes are removed with one ligature still attached and immersed in toto in 3% buffered paraformalin or in 2% glutaraldehyde. These lung lobes are sectioned for light and electron microscopy (including scanning electron microscopy) after 24 hours of fixation. The above procedure permits the performance of functionally relevant morphometric studies which can then be meaningfully correlated with the biochemical and functional parameters.

C. Coagulation and other hematologic changes in endotoxin shock with or without steroid treatment.

Our previous studies have indicated that continuous endotoxin infusion in primates is a reliable and clinically revelant model for the study of the mechanism and treatment of prolonged endotoxin shock. Using this model we have been able to provide evidence suggesting that glucocorticoids inhibit endotoxin induced: (a) sequestration, degranulation and fragmentation of the

leukocytes in the pulmonary microcirculation, (b) marked endothelial damage of the pulmonary microvasculature in association of interstitial edema, multifocal swelling of the type I epithelium and congestive atelectasis and (c) early development of diffuse microthrombosis in the sinusoids of the liver (and spleen) as well as centrilobular liver necrosis (3,4). These findings suggest that glucocorticoids inhibit certain endotoxin induced blood-vascular reactions which appear to play an important role in the development of pulmonary and other systemic complications in endotoxin shock. The present study was undertaken to evaluate the effects of endotoxin with or without steroid treatment on coagulation and other hematologic profiles. Such studies have been previously performed in primates but only following bolus endotoxin infusion which is an unsatisfactory model for the study of the mechanism and treatment of prolonged endotoxin shock.

Initially we investigated the hematologic changes induced by continuous endotoxin infusion (10 mg/kg/hr.) versus bolus endotoxin infusion (10 mg/kg injected over a 10 min. period). It should be mentioned that all animals that received bolus endotoxin injection rapidly developed hypotension and at the time of sacrifice (3 hrs.) the blood pressure averaged about 30% of the control blood pressure. By contrast, animals infused with endotoxin at a constant rate showed only a transient and relatively mild hypotensive response during the initial 3 hr. period, although they received larger amounts of endotoxin (Fig. 1). A major difference in the hematologic response between the two models was the finding that in the bolus model factor XII dropped rapidly within 30 min. after endotoxin infusion (Table II). By contrast in the continuous infusion model the level of factor XII actually increased after 30 min. but thereafter demonstrated gradual but progressive drop. All other hematologic and coagulation parameters were essentially the same in both models during the first 3 hr. period after the onset of endotoxin injection (Table II-IV). These findings suggest that the severe hypotensive response induced by bolus endotoxin is directly related to the rapid activation of factor XII.

In the continuous infusion model with or without steroid treatment the following hematologic changes were observed (Tables III-IV and Figs. 2-8).

(1) Leukocytes: In monkeys infused with endotoxin only the leukocytes demonstrated marked drop during the first three hours followed by rebound progressive increase thereafter. At 10 hours the leukocyte count had reached the pre-infusion level and at 16-18 hours the animals showed slight leukocytosis. By contrast steroid treated animals showed only mild drop in the leukocyte count at 30 min. Thereafter there was a sharp increase and at 6 hours the leukocyte count ($1000/mm^3$) was 22.5 ± 5.6 (baseline value 10.3 ± 1.8). In addition further progressive increase in the leukocyte count was noted throughout the 24 hour experimental period.

(2) Platelets and fibrinogen: They both demonstrated progressive drop in the continuous infusion model, but this drop was significantly less prominent in animals treated with steroids. (3) Coagulation factors: At 30 min. no significant changes were noted. After 3 hours factors V, VIII, and XII demonstrated progressive decrease while the drop of factor VII was marked only at 16-18 hours. Treatment with glucocorticosteroids significantly inhibited the drop of factor VIII but had no statistically significant affect on factor V and XII. (4) Plasminogen and plasmin activities. At present we have only results from one pair of monkeys one of which received 10 mg endotoxin/kg/hr and the other Ringer's lactate (control).

Plasminogen and plasmin activities were determined according the method by Silverstein (17). Progressive hyperplasminemia in association with decrease in the concentration of plasminogen were noted in the endotoxin-treated animal (table V).

The blood-vascular response to endotoxemia or septicemia can be best defined as an intravascular inflammatory reaction leading to margination and degranulation of leukocytes, activation of the coagulation and fibrinolytic systems (DIC) as well as activation of complement and kallikrein-bradykinin systems (5-16). In addition, there is evidence that activation of factor XII plays an initiating role in the activation of some of the above complex interactions which along with other neurohormonal changes eventually result in failure of the terminal microcirculation (shock). Therefore, a rational approach to the treatment of endotoxic shock is the utilization of drugs which inhibit vascular injury due to either sequestration and degranulation of leukocytes or excessive activation of the coagulation, fibrinolytic, complement and kallikrein-kinin systems. In this respect, our experimental studies suggest that glucocorticoids have a definite place in the treatment of endotoxin shock since they were found to inhibit the rapid development of DIC, sequestration and degranulation of leukocytes as well as pulmonary vascular injury and associated edema and congestive atelectasis of the lung. However, glucocorticoids were found to have no significant effect on the endotoxin induced progressive decrease in the blood levels of factors V and XII. In this connection it is of interest to mention that while a rapid and marked drop in the blood level of factor XII was found following bolus endotoxin infusion a more gradual but progressive decrease was invariably the case in the continuous infusion model for prolonged shock. This finding may explain the failure of previous investigators (18) to detect significant therapeutic effects with the glucocorticosteroids given prior to bolus endotoxin infusion.

D. The effects of epsilon amino caproic acid (EACA) and trasylol on endotoxin shock.

The rational for testing the possible role of these drugs in endotoxin shock is based on the evidence that excessive activation of the fibrinolytic and kallikrein-bradykinin systems apparently occurs in endotoxic shock. Our experience with the above drugs is at present limited and the findings described below are still preliminary.

(1) EACA: Four pairs of monkeys were used. In each pair of monkeys endotoxin was infused continuously at a rate of 10 mg/kg/hr, but one monkey from each pair also received EACA 50 or 100 mg/kg/hr. The monkeys were killed in pairs at 6 hours and 16 hours after the onset of endotoxin injection. The results that we have obtained thus far suggest that this drug does not significantly alter endotoxin induced hematologic changes but it does appear to inhibit margination of the leukocytes in the pulmonary vascular bed during the early stages of shock. These changes were associated with significant preservation of the endothelial integrity. In addition there was evidence that EACA inhibited formation of fibrinous deposits in the microcirculation of the liver and spleen possibly because the drug promoted endothelial preservation. We are currently performing studies designed to evaluate whether hematological

changes occur following in vitro interaction of EACA with normal, citrated plasma (pooled). Our preliminary findings suggest that EACA at concentrations of 0.4 mg/ml to 25 mg/ml does not result in any apparent alteration of basic coagulation profiles (PT, PTT, TT, fibrinogen), levels of various blood factors (II, V, VII, VIII, IX, X and XII) or the plasminogen-plasmin system. However, EACA supplementation was found to induce dispersion of platelets at a concentration of 50 mg/ml of platelet rich plasma. We may speculate, therefore, that the observed EACA-induced inhibition of fibrin deposition in the liver sinusoids is, in part, related to the effects of this drug on platelets. Further studies are in progress.

(2) Trasylol. This basic polypeptide derived from bovine lung is a polyvalent enzyme inhibitor which inactivates a variety of proteolytic enzymes including kallikrein, plasmin, plasmin activator, trypsin, chymotrypsin and katepsin D. In addition, trasylol was found to possess anticoagulant properties which appear to be confined to the early stages of coagulation. Trasylol is eliminated rapidly via the kidneys and has a half life of about 120 minutes. Clinically, trasylol has been extensively used in Europe primarily to combat pancreatitis and postoperative hyperfibrinolytic hemorrhages. In addition, there is some clinical and experimental evidence that the drug has considerable therapeutic value in various forms of shock presumably due to inhibition of excessive proteolysis including liberation of kinins and normalization of microcirculation. It should be emphasized, however, that the precise therapeutic effect of trasylol in shock is still unknown. Moreover, there is virtually no information relating to the pharmacological effects of this drug on primate models.

The following pilot experiments have been performed. Four pairs of monkeys were infused with endotoxin (10 mg/kg/hr) but one animal from each pair was also given trasylol, 5,000 KIU/kg/hr. The animals were sacrificed 6 or 10 hours after the onset of endotoxin infusion. We have not as yet completed evaluation of the above pilot experiments but our preliminary findings suggest that the dose-response of these drugs should be carefully evaluated because one of the trasylol treated animals developed disseminated intravascular thrombosis. We are currently studying the in vitro effect of trasylol supplementation on citrated pooled plasma in order to meaningfully evaluate the in vivo therapeutic value of this drug. Our preliminary in vitro studies suggest that following incubation of citrated plasma with added trasylol (at concentrations 100 KIU/ml plasma) at 37° C for 30 min, trasylol inhibits plasminogen. In addition, trasylol was found to prolong PTT suggesting that it may interfere with the intrinsic pathway of coagulation. It was further noted that trasylol interfered in the assays of factors VIII, IX and XIII. Further studies are in progress.

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Table Ia

BIOCHEMICAL STUDIES OF THE SURFACTANT SYSTEM IN ONE MONKEY INFUSED 10 HOURS WITH 10 mg. ENDOTOXIN/Kg/Hr. (Case No. 85 EN) AND IN THE PAIRED CONTROL (Case No. 86 C) INFUSED WITH RINGER'S LACTATE SOLUTION

Case Number	Total Surfactant PL(mg)/Left Lung		PL/Protein of Surfactant Fractions	Distribution of PL in Extracellular (E) and Intracellular (I) Surfactant						
				Sph	PC	PS	PI	PE	PG	UN
85 EN	44.9	E	4/1	0.8	84.9	0.7	6.8	2.2	4.0	0.6
		I	3/1	17.5	40.9	22.4	0.9	18.3	0	0
86 C	47.3	E	23/1	0.6	80.9	0.3	11.9	3.9	0.8	1.6
		I	3/1	20.5	49.6	6.8	1.3	21.5	0.3	0

FATTY ACID COMPOSITION OF PHOSPHATIDYL CHOLINE OF SURFACTANT FRACTIONS - PERCENT OF TOTAL FATTY ACIDS

		14:0	16:0	16:1	18:0	18:1	18:2	20:4	22:0
85 EN	E	1.6	63.4	5.3	6.9	12.5	7.8	2.3	0.2
	I	0.6	39.9	2.0	16.5	19.6	12.4	8.0	1.0
86 C	E	1.7	63.4	5.2	7.6	14.3	6.5	1.1	0.2
	I	0.3	37.1	2.0	18.3	20.7	10.3	10.1	1.1

Abbreviations used in the table:

E = extracellular (alveolar) surfactant; I = intracellular surfactant

PL = phospholipid

Sph = sphingomyelin; PC = phosphatidyl choline; PS = phosphatidyl serine; PI = phosphatidyl inositol

PE = phosphatidyl ethanolamine; PG = phosphatidyl glycerol

UN = unidentified phospholipids

Table Ib

Case Number		Total Surfactant PL(mg)/Left Lung
85 EN	E[a]	14.0
	I[a]	30.9
86 C	E	29.7
	I	17.6

a - E = extracellular (alveolar) surfactant; I = intracellular surfactant

TABLE II. Hematologic Data in Monkeys Infused With Bolus Endotoxin, 10 mg/kg, Infected over a 10 min. period (mean±SEM)

Parameter	Baseline	Minutes After Endotoxin Injection	
		30	180
Leukocytes ($\times 1000/mm^3$)	7.6±1	2.15±0.4	2.9±0.6
Platelets ($\times 1000/mm^3$)	355±73	234±11	234±38
Fibrinogen (mg/100 ml)	180±23	176±24	150±19
Factors (% of Control)			
II	140±16	111±21	99±14
V	139±9	136±32	94±21
VII	92±5	93±7	72±10
X	108±3	97±1	83±5
VIII	160±40	107±17	80±13
IX	115±20	94±1	96±16
XII	183±26	94±6	89±9
Number of Animals	3	3	3

TABLE III.

Hematologic Data in Monkeys Infused with Endotoxin at a Rate of 10 mg/kg/hr (mean ± SEM)

			Time After Onset of Endotoxin Infusion					
Parameter	Exper. Group*	Baseline	30 min.	3 hrs.	6 hrs.	10 hrs.	16-18 hrs.	22-24 hrs.
Hgb	(A)	13.1 ± 0.35	12.5 ± 0.3	12.8 ± 0.3	12.8 ± 0.3	13.2 ± 0.4	13.7 ± 1.0	-
	(B)	13.2 ± 0.24	12.9 ± 0.4	13.2 ± 0.2	13.1 ± 0.2	12.5 ± 0.4	-	12.6 ± 0.6
Hct	(A)	41.1 ± 1.74	38.5 ± 1.1	41.0 ± 1.4	39.8 ± 0.9	40.4 ± 1.1	42.3 ± 2.7	-
	(B)	41.5 ± 0.74	40.6 ± 1.4	41.2 ± 1.1	41.2 ± 0.8	39.4 ± 1.3	-	40.7 ± 2.8
Leukocytes ($x1000/mm^3$)	(A)	10.3 ± 1.8	4.7 ± 1.6	3.2 ± 0.6	7.2 ± 2.4	9.6 ± 3.3	13.4 ± 4.8	-
	(B)	10.8 ± 1.5	7.2 ± 1.2	10.2 ± 2.1	22.5 ± 5.6	25.3 ± 10.1	-	26.1 ± 7.4
Platelets ($x100\ mm^3$)	(A)	346 ± 25	312 ± 22	271 ± 29	141 ± 27	118 ± 26	30 ± 4	-
	(B)	347 ± 17	309 ± 45	268 ± 24	229 ± 23	180 ± 72	-	49 ± 16
Fibrinogen (mg/100 ml)	(A)	165 ± 10	166 ± 9	121 ± 23	97 ± 11	80 ± 5	52(30-74)**	-
	(B)	170 ± 20	163 ± 19	133 ± 19	120 ± 3	116 ± 9	-	108 ± 16
Number of Animals (n)	(A)	8	5	5	5	4	3	-
	(B)	8	3	4	5	3	-	3

*A, Endotoxin group; B, Endotoxin + steroid group
**Data from 2 animals

TABLE IV.

Coagulation Factors in Monkeys Infused with Endotoxin at a Rate of 10 mg/kg/hr (mean ± SEM)

			Time After Onset of Endotoxin Infusion					
Parameter	Exper. Group*	Baseline	30 min.	3 hrs.	6 hrs.	10 hrs.	16-18 hrs.	22-24 hrs.
II	(A)	108 ± 7	110 ± 10	94 ± 3	91 ± 15	90 ± 18	63 ± 13	-
	(B)	107 ± 11	99 ± 1	99 ± 2	85 ± 3	84 ± 1	-	56 ± 8
V	(A)	117 ± 20	108 ± 30	52 ± 15	40 ± 12	41 ± 14	33 ± 7	-
	(B)	94 ± 4.7	93 ± 7	56 ± 10	44 ± 8	57 ± 7	-	35(5-65)**
VII	(A)	100 ± 21	130 ± 31	85 ± 15	80 ± 11	72 ± 4	26 ± 4	-
	(B)	103 ± 12	89 ± 11	74 ± 5	70 ± 6	61 ± 11	-	45 ± 3
X	(A)	88 ± 13	109 ± 11	79 ± 13	66 ± 12	71 ± 6	31 ± 6	-
	(B)	91 ± 14	98 ± 2	85 ± 13	73 ± 9	73 ± 3	-	17 ± 2
VIII	(A)	97 ± 13	105 ± 2	69 ± 13	27 ± 6	25 ± 5	5 ± 1	-
	(B)	100 ± 8	99 ± 15	86 ± 7	68 ± 14	55(32-78)**	-	17.2
IX	(A)	106 ± 8	104 ± 16	83 ± 21	57 ± 9	39 ± 12	24 ± 2	-
	(B)	105 ± 9	100 ± 1	82 ± 5	74 ± 3	58 ± 10	-	32(20-45)**
XII	(A)	127 ± 16	151 ± 23	71 ± 11	65 ± 12	45 ± 6	36 ± 5	-
	(B)	120 ± 19	115 ± 45	103 ± 22	98 ± 31	57 ± 4	-	43 ± 7
Number of Animals (n)	(A)	6	3	3	4	3	3	-
	(B)	5	3	3	3	3	-	3

*A, Endotoxin group; B, Endotoxin + steroid group
**Data from 2 animals

TABLE V

DETERMINATION OF PLASMIN AND PLASMINOGEN ACTIVITIES

IN CONTROL AND ENDOTOXIN MONKEY

Time	Control (Ringer's Lactate, 9 ml/hr)		Endotoxin (10 mg/kg/hr)	
	Plasmin (% total)	Plasminogen (% Control)	Plasmin (% total)	Plasminogen (% of Control)
0	26	100	23	100
1 hour	26	94	25	82
6 hours	23	89	62	61
12 hours	37	84	72	56

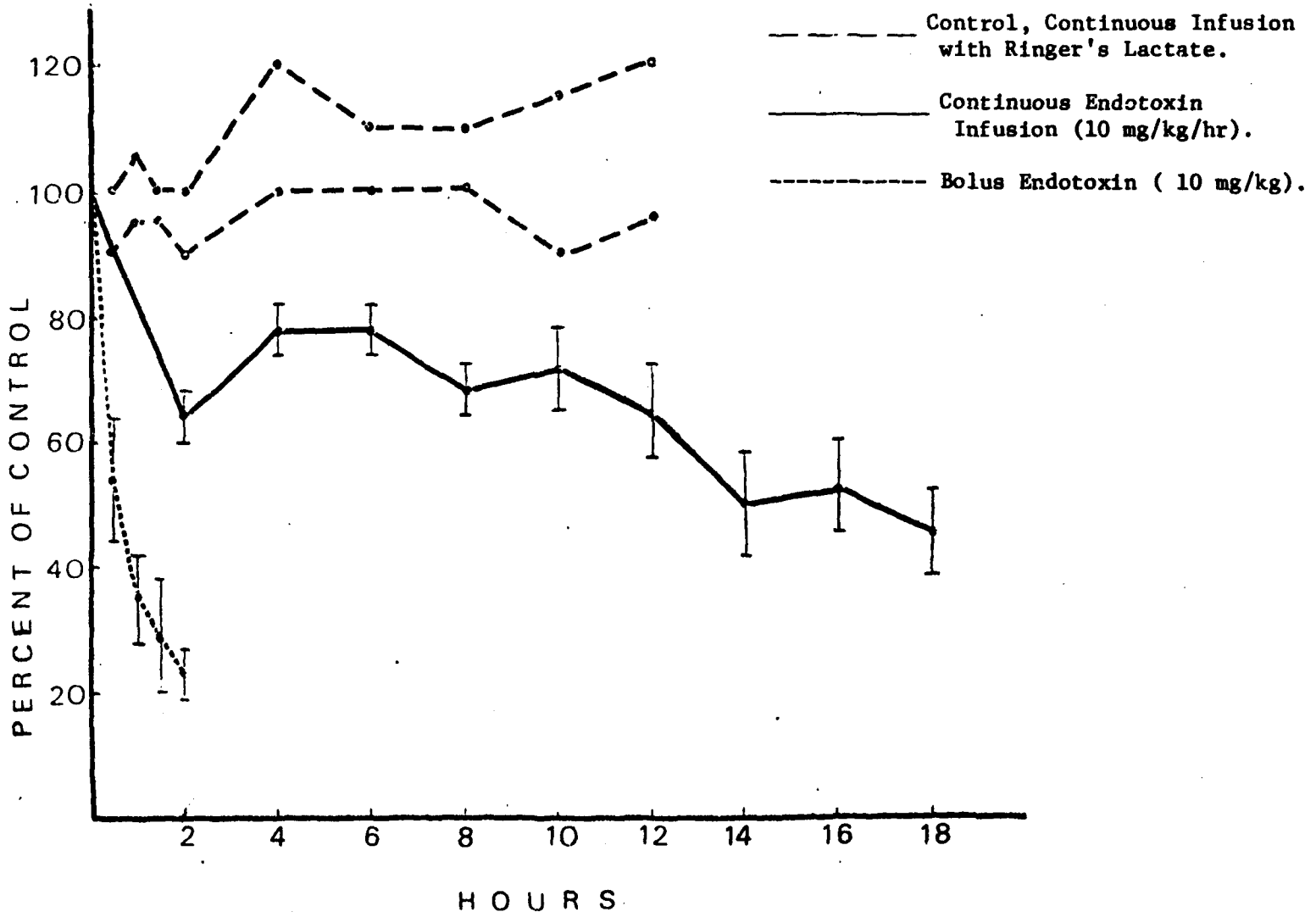
Control, Continuous Infusion with Ringer's Lactate.
Continuous Endotoxin Infusion (10 mg/kg/hr).
Bolus Endotoxin (10 mg/kg).
PERCENT OF CONTROL
120
100
80
60
40
20
2
4
6
8
10
12
14
16
18
HOURS

Figure 1.

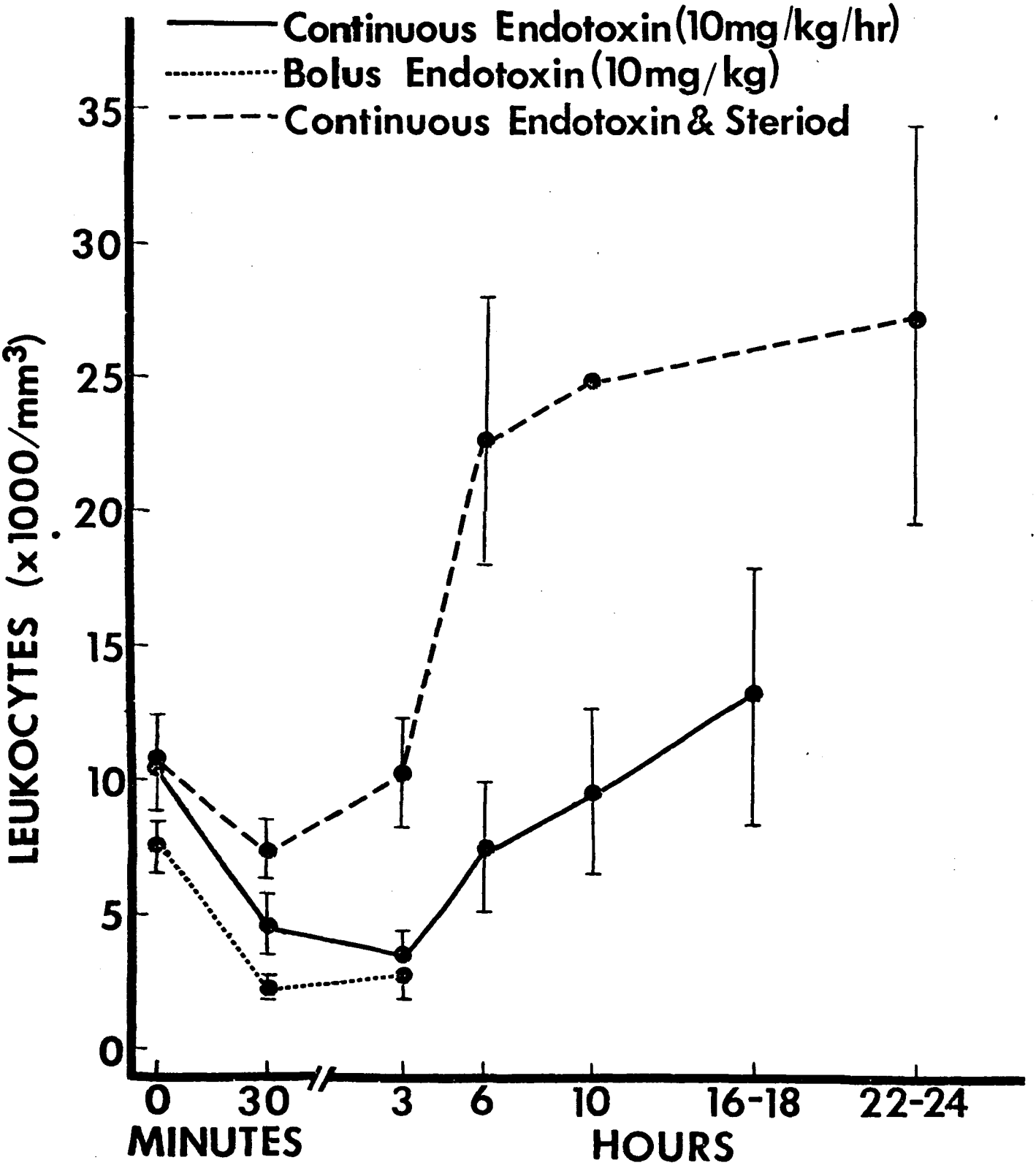
Continuous Endotoxin (10mg/kg/hr)
Bolus Endotoxin (10mg/kg)
Continuous Endotoxin & Steriod
LEUKOCYTES (x1000/mm³)
35
30
25
20
15
10
5
0
0
30
3
6
10
16-18
22-24
MINUTES
HOURS

Figure 2.

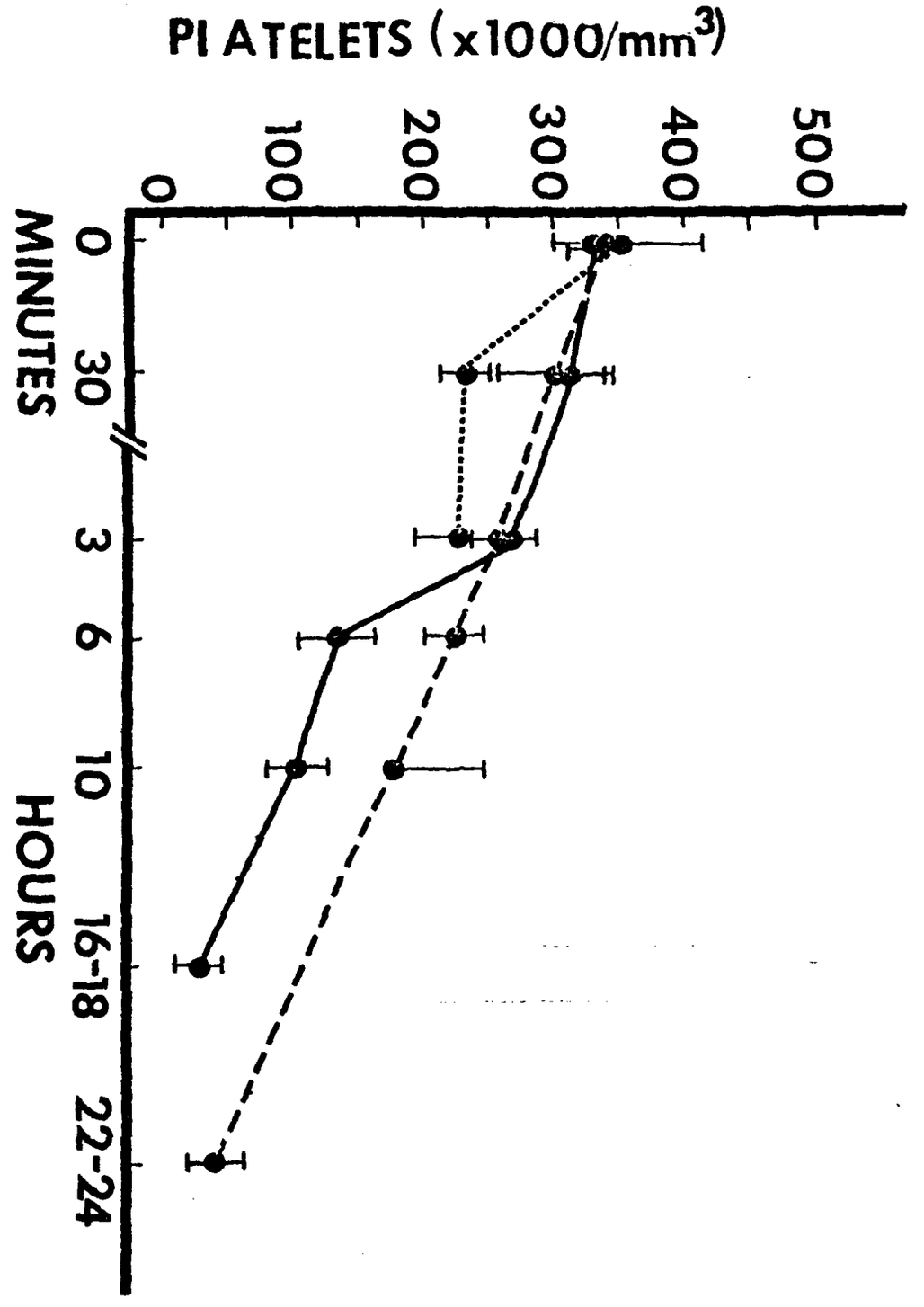
PLATELETS (x1000/mm3)
0
100
200
300
400
500
MINUTES
0
30
HOURS
3
6
10
16-18
22-24

Figure 3.

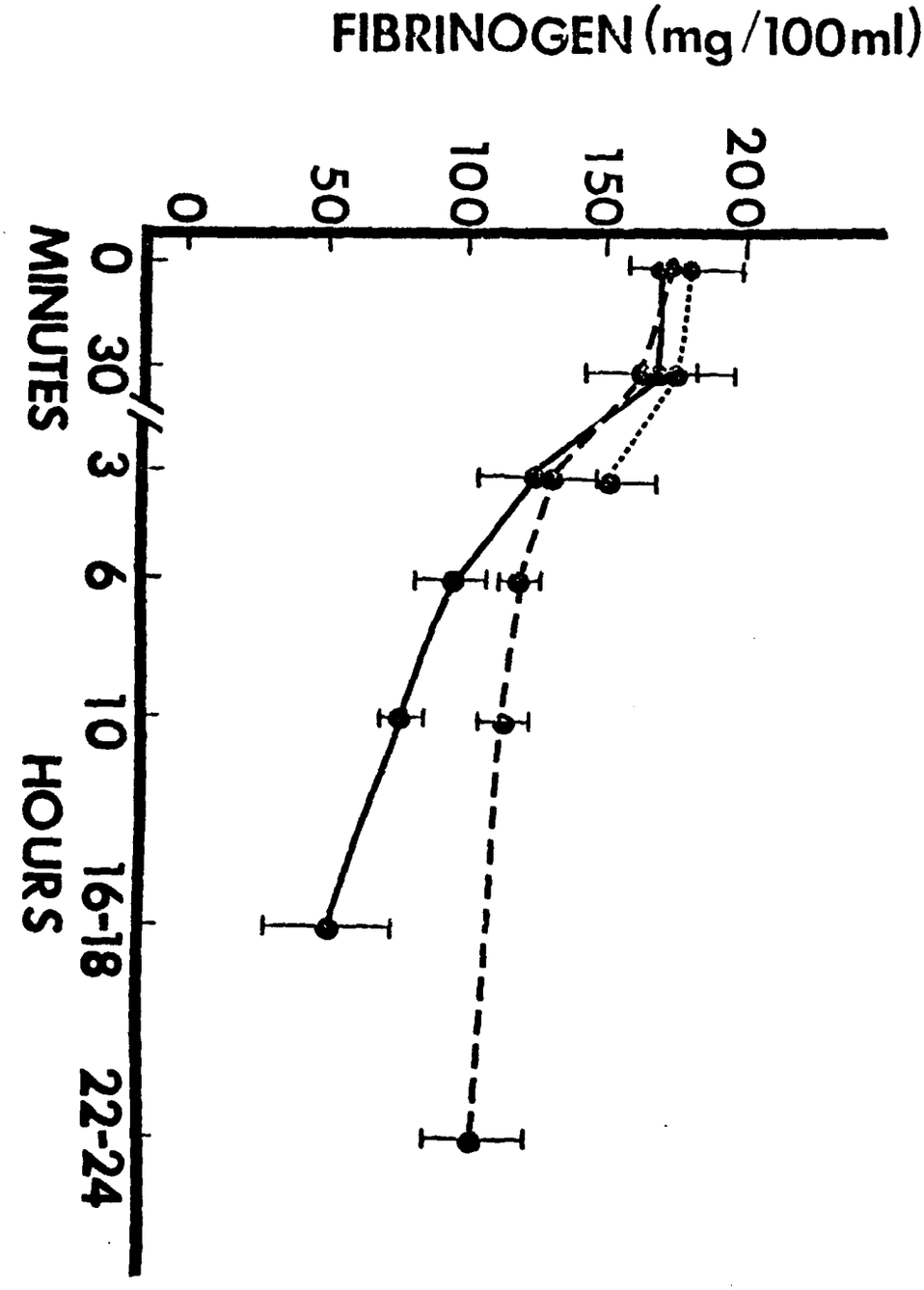
FIBRINOGEN (mg/100ml)
0
50
100
150
200
0
30
3
6
10
16-18
22-24
MINUTES
HOURS

Figure 4.

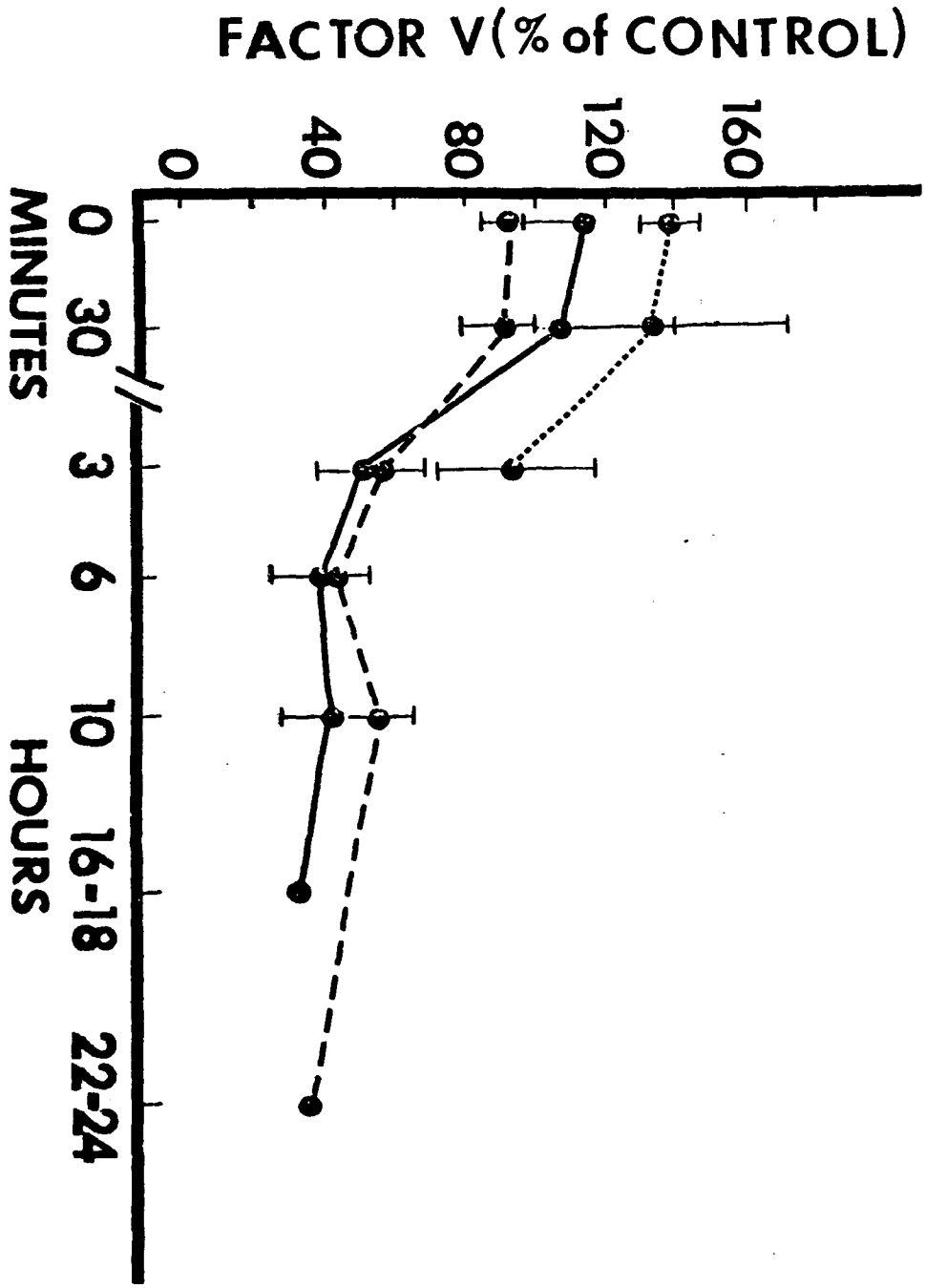
FACTOR V(% of CONTROL)
0
40
80
120
160
MINUTES
0
30
HOURS
3
6
10
16-18
22-24

Figure 5.

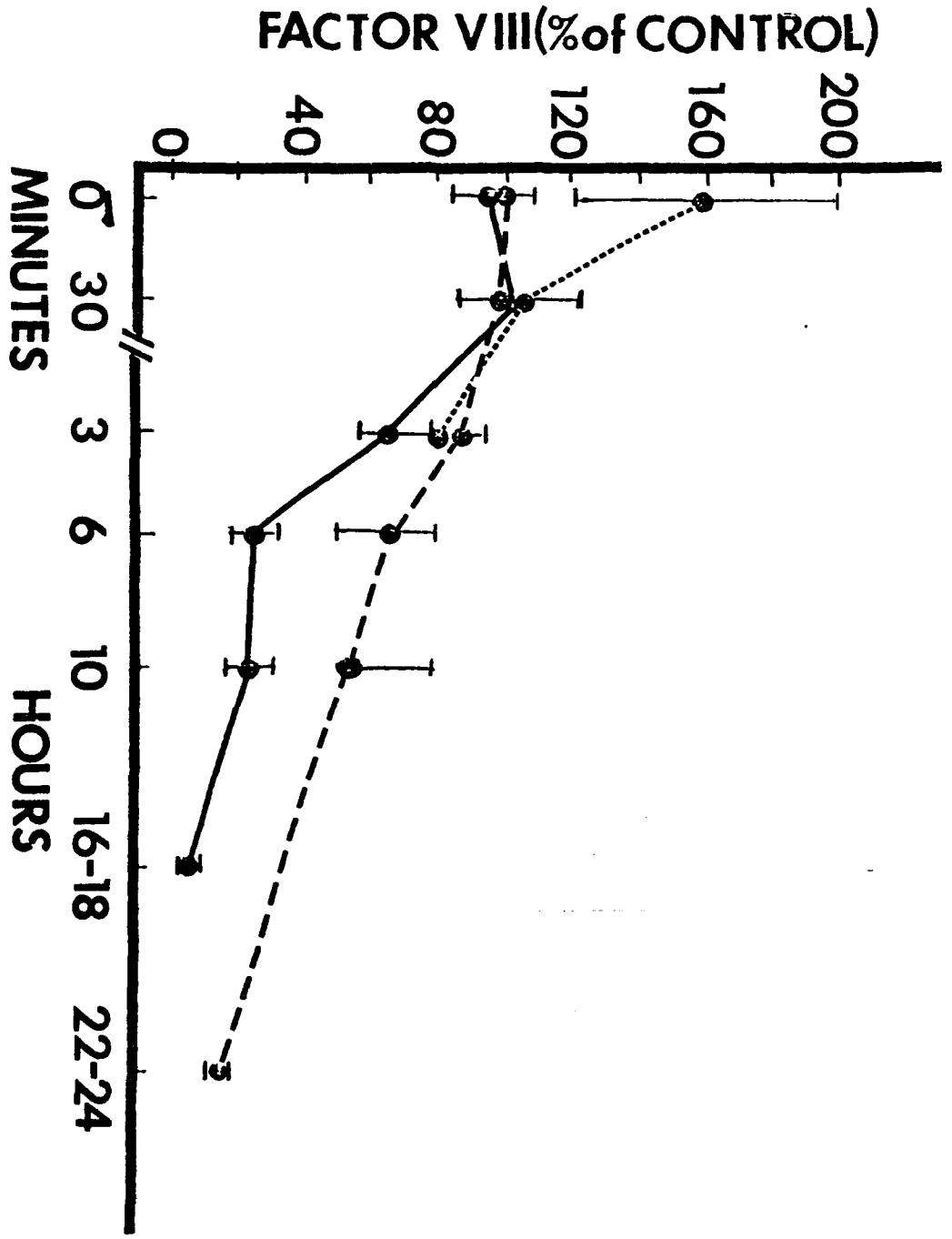
FACTOR VIII(%of CONTROL)
0
40
80
120
160
200
0
30
3
6
10
16-18
22-24
MINUTES
HOURS

Figure 6.

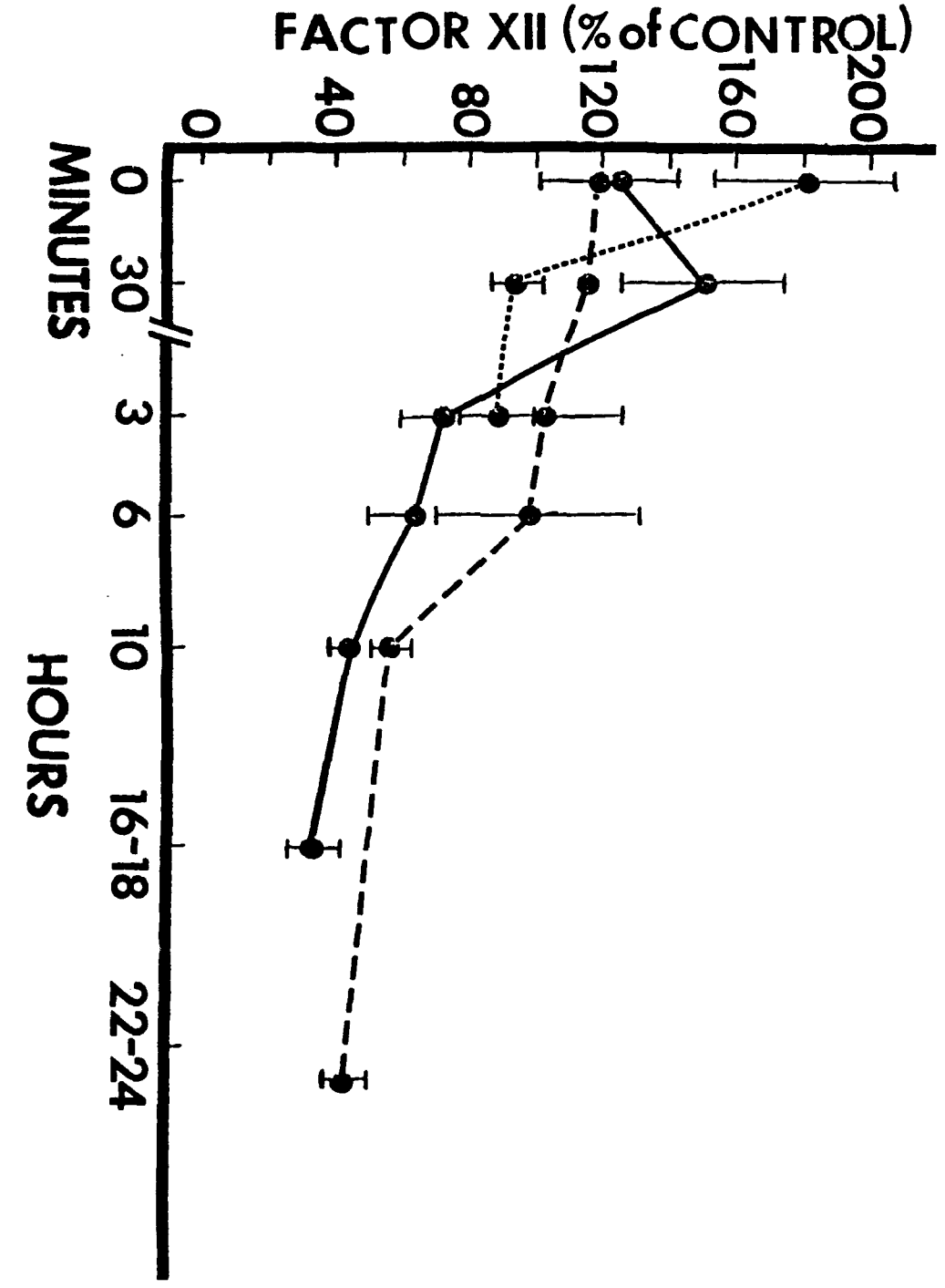
FACTOR XII (% of CONTROL)
0
40
80
120
160
200
MINUTES
0
30
HOURS
3
6
10
16-18
22-24

Figure 7.

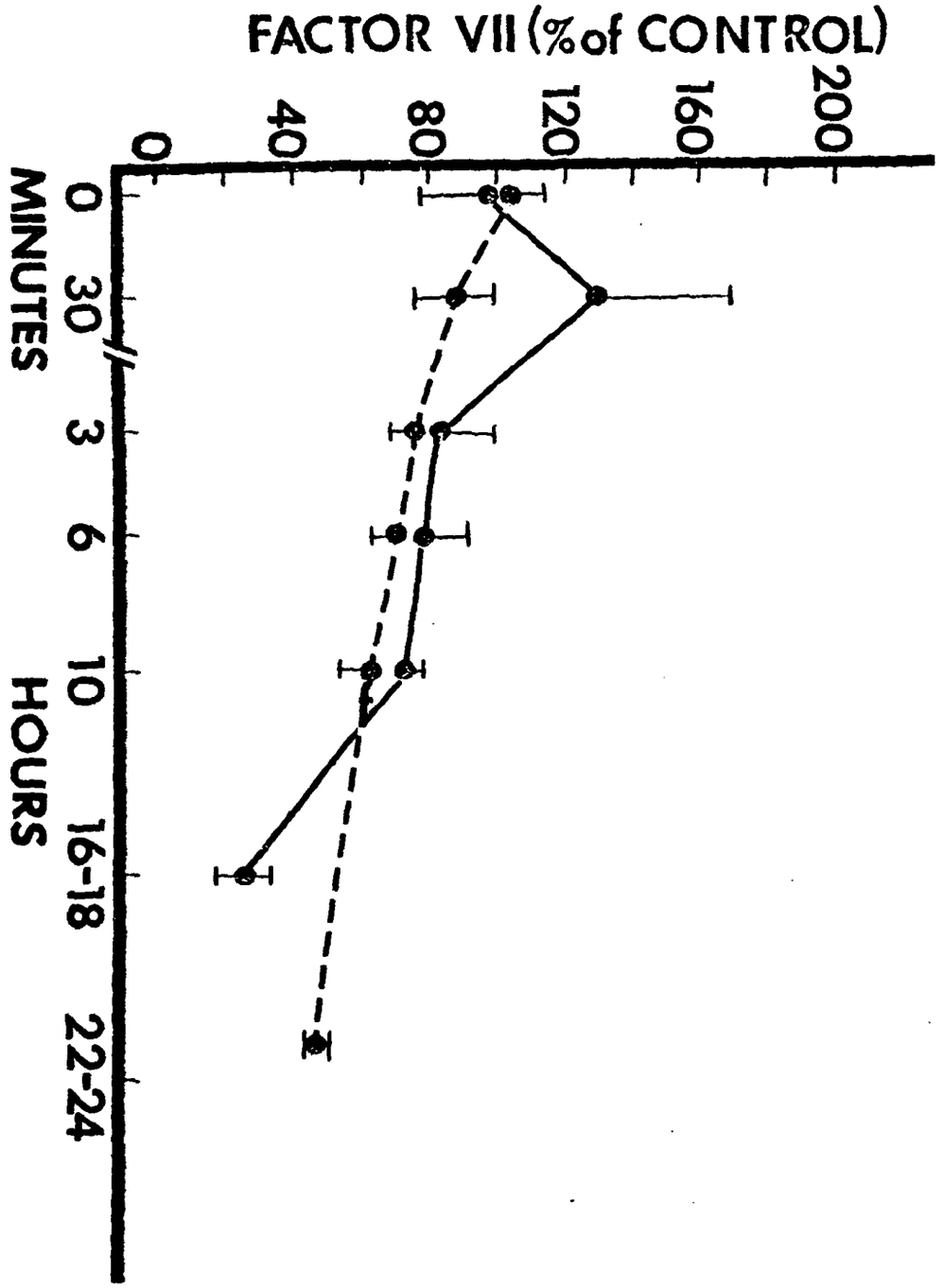
FACTOR VII (% of CONTROL)
0
40
80
120
160
200
0
30
3
6
10
16-18
22-24
MINUTES
HOURS

Figure 8.

Studies on pharyngeal aspirates of full-term infants and tracheal aspirates of newborn infants maintained on respirators.

Pharyngeal aspirates obtained at time of delivery from 20 normal full-term infants were collected. Following centrifugation at 480 g for 5 minutes the supernatants were used to prepare 5 ml gradients. Following centrifugation a narrow visible band of white cloudy or particulate material was seen about 1.5 cm from the top of the tube. To confirm that the visible band of material corresponded to the phospholipid-rich surfactant band, as had been found with rabbit lung and 60 ml gradients, fractions were collected from several gradients of pharyngeal aspirates. The fractions were analyzed for phospholipid and protein content and as shown in figure 1, a distribution similar to that found in gradients of rabbit lung washings was found. Therefore, in later experiments designed to study the surfactant of the pharyngeal aspirates, the visible band of material was removed from gradients and the remaining fractions were discarded. Surface activity of the surfactant isolated from some of the pharyngeal aspirates was measured in a modified Langmuir-Wilhelmy surface balance. The material was surface active with samples containing 200 μg of phospholipid reducing the minimum surface tension to 13 dynes/cm, giving a stability index of 1.1 and a hysteresis loop with an area of 1.3 square inches. Biochemical studies of the surfactant isolated from pharyngeal aspirates of full-term newborns revealed that PC* was the major phospholipid present accounting for 83% of the total phospholipid (Table I). PG* was the second most abundant phospholipid with smaller amounts of Sph*, PS, PI and PE also being detected. The fatty acid composition of the PC showed, as expected, a relatively high per cent of palmitic acid (Table I), with much lower amounts of other commonly found fatty acids. These values for normal human newborn surfactant are currently useful as control values for the study of developing lung surfactant isolated from amniotic fluid at various gestational ages and from tracheal aspirates of infants maintained on respirators.

Tracheal aspirates routinely taken at regular intervals from intubated premature infants as a part of the therapeutic procedure to keep the airways patent, are collected every 24 hours from the neonatal intensive care unit. Within the last 14 months, samples have been obtained from 113 infants with as many as 57 serial samples obtained per patient (Table II). Initially samples were centrifuged at 480 g and the pellets and supernatant separately frozen at -70°C. In the early studies only supernatants which did not appear to be contaminated with blood were used directly for biochemical studies. More recently, surfactant purified by the density gradient method described in section c-III has been isolated from the supernatants, which allows use of any sample, even those grossly contaminated with blood. Also, the density gradient purification we are now using permits elimination of the first low speed centrifugation allowing more complete recovery of the relatively small amounts of surfactant present in these aspirates. The first investigations using the tracheal aspirates were determinations of the lecithin to sphingomyelin ratios of clean appearing supernatants. In all cases studies, even very premature infants of less than 1000 gm, the L/S ratios of the tracheal aspirates were quite high and were of no diagnostic value in predicting the clinical course of the infant. However, a careful investigation of the fatty acid composition of PC in serial samples from three infants has revealed significant changes (Tables III, IV, V). In the first case studied (Table III), tracheal aspirate supernatants were used. In the second case (Table IV), tracheal aspirate supernatants and purified surfactant were compared. As can be seen in Table IV on day 31, relatively small differences are seen when one is careful to select only aspirates not contaminated with blood. In the third case surfactant was purified from all samples before separation of PC and

analysis of fatty acids. This is now our routine procedure since it permits use of all samples collected. Both baby girl R and Baby boy M (Tables III and IV) were diagnosed as having HMD and at 3 to 4 days of life have a PC fatty acid distribution quite different from healthy full term newborns with the palmitic acid content being lower and the oleic acid content higher. Baby girl Z, also very premature (Table V) but apparently without HMD, had at 3 days, a PC fatty acid composition more closely resembling the normal full term newborn controls. Baby boy M, who did not survive, revealed some interesting changes in the less abundant fatty acids with increasing postnatal age. Palmitoleic acid (16:1) decreased from 8% at 4 days to 0.5% at 39 days. An opposite change was noted in linoleic acid (18:2) which increased from 1.5% to 16.2% at 39 days. Similar changes but of a somewhat lesser magnitude were seen in the fatty acids of PC in Baby girl R who survived. The importance of these findings cannot as yet be evaluated, since careful consideration of the methods of treatment and clinical course is necessary as well as the study of a much larger number of cases.

*Abbreviations used in the text:

PC - Phosphatidyl choline
PG - Phosphatidyl glycerol
Sph - Sphingomyelin
PS - Phosphatidyl serine
PT - Phosphatidyl inositol
P - Ethanolamine

Table I

BIOCHEMICAL STUDIES OF SURFACTANT ISOLATED FROM FIRST ASPIRATES OF FULL TERM INFANTS

A. Distribution of Phospholipids

Percent of Total Phospholipids[a]

LPC	Sph	PC	PS	PI	PE	PG
0	0.2	83.4	3.3	2.3	2.2	8.6

B. Major[b] Fatty Acids of Phosphatidylcholine

Percent of Total Fatty Acids[c]

14:0	16:0	16:1	18:0	18:1	18.2	20:4
3.1 (0.7)	71.7 (1.1)	6.0 (0.5)	4.6 (0.4)	9.6 (0.7)	2.7 (0.3)	2.3 (0.3)

a-Values are average of samples from 4 infants.
b-A number of other fatty acids were found when the sample size was large. These included odd carbon fatty acids, 13:0, 15:0 and 17:0 and some unidentified fatty acids with retention times greater than 20:0. However, in tracheal aspirates these fatty acids were always less than 1% of the total fatty acids.
c-Values are average of samples from 13 infants. The numbers in parentheses indicate standard error of the mean.

Table II

TRACHEAL ASPIRATES FROM NEWBORNS MAINTAINED ON RESPIRATORS

Group A. Infants With Non-Pulmonary Diseases.

Sample Number	Gestational Age (wks)	Weight (gm.)	Number of Aspirates Collected	Clinical Diagnosis	Outcome	Pathological Diagnosis
92	32	1000	1	Sepsis	S[a]	
73	32	1700	3	Erythro-blastosis	S	
33	33	1780	2	Cong. Anomalies	S	
29	33	1940	31	Polycystic Disease	S	
48	34	1760	2	Intraut. Growth Retardation	S	
38	Term	2255	2	Meningomyelo-cele	D-7[b]	Cong. Anomalies
21	Term	2540	4	Sepsis	S	
64	Term	2580	1	Cong. Anomalies	D-1	Cong. Anomalies
26	Term	3000	4	Ileal Atresia	S	
60	Term	3120	2	Sepsis	S	
63	Term	3320	12	Intraut. Asphyxia	S	
30	Term	3500	1	Sepsis	S	
52	Term	3560	1	Meningomyelo-cele	S	
27	Term	4120	1	Cong. Heart Disease	S	
7	Term	4200	2	Omphalocele	S	
59	Term	4200	2	Cong. Heart Disease	S	

Group B. Infants With Pulmonary Disease Other Than RDS.

Sample Number	Gestational Age (wks)	Weight (gm.)	Number of Aspirates Collected	Clinical Diagnosis	Outcome
96	28	880	4	Apnea	S
12	28	1038	18	Wilson-Mikity Syndrome	S
37	31	1850	19	Tracheoesophageal Fistula	S
43	34	2780	10	Aspiration Pneumonia	S
95	Term	2620	6	Bilateral Pneumothorax	S
84	Term	2800	3	Aspiration Pneumonia	S
87	Term	3100	2	Meconium Aspiration	S
81	Term	3200	2	Aspiration Pneumonia	S
51	Term	3240	7	Meconium Aspiration	S
40	Term	3500	2	Aspiration Pneumonia	S
83	Term	3500	3	Amniotic Fluid Aspiration	S
102	Term	3620	1	Aspiration Pneumonia	S
23	Term	4240	4	Aspiration Pneumonia	S

a. S = survived
b. D = died - number of days after birth

Table II (Continued)

Group C. Infants With No Apparent Disease Except Prematurity.

Sample Number	Gestational Age (wks)	Weight (gm.)	Number of Aspirates Collected	Outcome	Pathological Diagnosis
28	23	790	11	D-8	No Autopsy
34	26	720	28	S	
82	28	950	5	S	
10	28	1040	1	S	
101	31	1080	3	S	
46	34	1800	3	S	
47	34	2020	4	S	

Group D. Infants With RDS.

Sample Number	Gestational Age (wks)	Weight (gm.)	Number of Aspirates Collected	Outcome	Pathological Diagnosis
44	24	590	1	D-1.5	Pulmonary Hemorrhage
16	24	680	5	S	
49	24	790	57	S	
25	24	840	2	D-20	No Autopsy
18	24	890	4	D-1	No Autopsy
1	25	830	3	D-7	Pneumonia
3	25	920	51	S	
71	26	890	3	D-3	No Autopsy
89	26	900	9	D-15	Aortic Thrombosis
97	27	800	2	D-4	Intraventricular Hemorrhage
78	27	960	1	D-2	HMD
79	28	1000	1	D-2	No Autopsy
5	28	1080	1	S	
77	28	1100	3	D-3	No Autopsy
2	28	1110	33	D-40	HMD
17	28	1160	2	D-2	No Autopsy
6	28	1480	3	S	
58	29	1100	14	S	
112	29	1170	4	D-5	HMD
41	29	1260	1	D-1	HMD
69	29	1600	1	D-2	HMD
54	30	720	3	D-1	HMD
103	30	1020	7	S	
11	30	1100	2	D-3	HMD
107	30	1100	2	D-10	No Autopsy
55	30	1180	11	S	
91	30	1200	2	S	
53	30	1300	2	D-1	HMD
39	30	1420	19	S	
68	30	1800	1	S	
93	31	1260	2	D-10	HMD
13	31	1400	21	S	
62	31	1500	3	D-5	No Autopsy
22	32	1200	22	S	
88	32	1660	4	S	
85	32	1680	1	D-4	HMD

Table II (Continued)

Group D. (Continued)

Sample Number	Gestational (Age (wks)	Weight (gm.)	Number of Aspirates Collected	Outcome	Pathological Diagnosis
35	32	1720	15	S	
14	32	1800	8	D-7	HMD
76	32	2000	5	S	
67	32	2160	5	S	
66	32	2400	12	S	
45	33	1420	12	D-10	HMD
19	33	1480	2	S	
98	33	1980	2	S	
106	33	2000	3	S	
109	33	2100	2	S	
74	33	2150	8	S	
94	33	2240	2	S	
100	34	1500	6	D-10	Pneumonia
104	34	1660	3	D-11	Aortic Thrombosis
15	34	1720	1	D-2	HMD
56	34	1800	11	S	
90	34	1800	3	S	
72	34	1860	1	D-3	HMD
57	34	1950	18	S	
4	34	1980	1	S	
24	34	2000	12	S	
65	34	2020	2	D-7	HMD
75	34	2200	9	S	
108	34	2200	8	D-20	No Autopsy
110	34	2320	3	S	
61	35	2540	5	S	
111	35	2580	2	S	
9	36	2200	8	S	
70	36	2320	1	D-2	HMD
42	36	2360	10	S	
36	36	2500	4	S	
86	36	2560	2	S	
8	36	2940	1	S	
20	36	3020	6	S	
50	37	2380	36	S	
99	37	2700	2	S	
32	38	2550	1	S	
105	38	3400	7	S	

Table III

FATTY ACIDS OF PHOSPHATIDYLCHOLINE

		% of Total Fatty Acids					
		14:0	16:0	16:1	18:0	18:1	18:2
Baby Girl R.	4 days	1.0	53.7	7.6	9.5	24.3	3.9
	25 days	4.5	57.7	3.5	7.2	13.8	13.3
	31 days	5.7	53.2	2.8	6.6	15.7	16.0
	33 days	7.9	55.1	5.3	8.1	12.1	11.5
	40 days	7.0	60.0	3.0	5.3	10.7	14.0

Baby Girl R., a premature infant of 25 weeks gestation, weighed 780 gm at birth and developed severe Grade 4 hyaline membrane disease. Endotracheal intubation and artificial ventilation were required for 44 days. Oxygen supplementation was stopped on the 58th day and the infant was discharged on the 105th day.

Tracheal aspirates were collected and following a low speed centrifugation, lipids were extracted from the supernatants. Phosphatidyl choline was separated by thin layer chromatography and the fatty acid composition of the PC was determined.

Table IV

FATTY ACIDS OF PHOSPHATIDYLCHOLINE

		% of Total Fatty Acids						
		14:0	16:0	16:1	18:0	18:1	18:2	20:4
Baby Boy M.	4 days (TA)	3.4	61.4	8.1	4.9	17.9	1.5	2.8
	5 days (S)	1.3	56.9	8.7	5.9	19.9	1.7	5.6
	10 days (TA)	2.7	61.2	7.1	5.5	16.9	1.3	5.3
	26 days (TA)	5.0	60.5	5.1	6.5	15.7	3.6	3.6
	31 days (TA)	3.7	55.2	1.9	9.2	12.8	13.3	3.8
	31 days (S)	2.1	55.4	2.2	13.0	13.3	12.6	1.4
	37 days (S)	3.5	61.6	1.5	9.6	9.5	13.0	1.3
	38 days (TA)	5.8	58.7	0.5	9.1	11.6	14.3	Not measured
	39 days (TA)	6.6	56.7	0.5	8.9	11.1	16.2	Not measured

Baby Boy M., a premature infant of 27 weeks gestation, weighed 1200 gm at birth and required endotracheal intubation and artificial ventilation shortly after birth. A clinical diagnosis of Grade 2-3 hyaline membrane disease was made. On the 21st day an increased need for oxygen supplementation occurred and the need for relatively high F_IO_2 ventilatory assistance remained unchanged until the 41st day when sudden deterioration in the patient's condition was followed by a fatal cardiac arrest.

Tracheal aspirates were collected and centrifuged at 500 g to remove cells and debris. In some instances the lipids were extracted directly from the supernatant and the fatty acid composition of the PC determined (indicated on the Table as TA). In other instances the supernatants were used to prepare density gradients for isolation of the lung surfactant fraction. The fatty acid composition of the PC of the purified surfactant is indicated in the table by S.

Table V

FATTY ACIDS OF PHOSPHATIDYLCHOLINE

		% of Total Fatty Acids						
		14:0	16:0	16:1	18:0	18:1	18:2	20:4
Baby Girl Z.	3 days	3.6	67.0	8.3	3.5	13.3	1.6	2.7
	11 days	0.2	59.8	6.7	6.8	23.2	0.4	2.9
	40 days	4.0	61.4	10.3	5.2	16.4	1.2	1.5
	45 days	3.2	65.4	12.1	2.9	14.2	1.0	1.2
	54 days	2.8	68.6	11.9	3.5	11.8	0.4	1.0

Baby Girl Z., a premature infant of 24 weeks gestation, weighed 790 gm at birth. Satisfactory oxygenation was achieved with F_IO_2 of 0.3, but progressive hypoxemia and apnea during the next 72 hours led to endotracheal intubation and institution of artificial ventilation with F_IO_2 of 0.8-0.9. The F_IO_2 was subsequently decreased with extubation and termination of respiratory support on the 74th day. The infant was discharged on the 121st day.

Tracheal aspirates were collected and following a low speed centrifugation lung surfactant was isolated from the supernatant by density gradient centrifugation. Lipids were extracted from the surfactant band, PC was separated from the lipid extract and the fatty acid composition of the PC was determined.

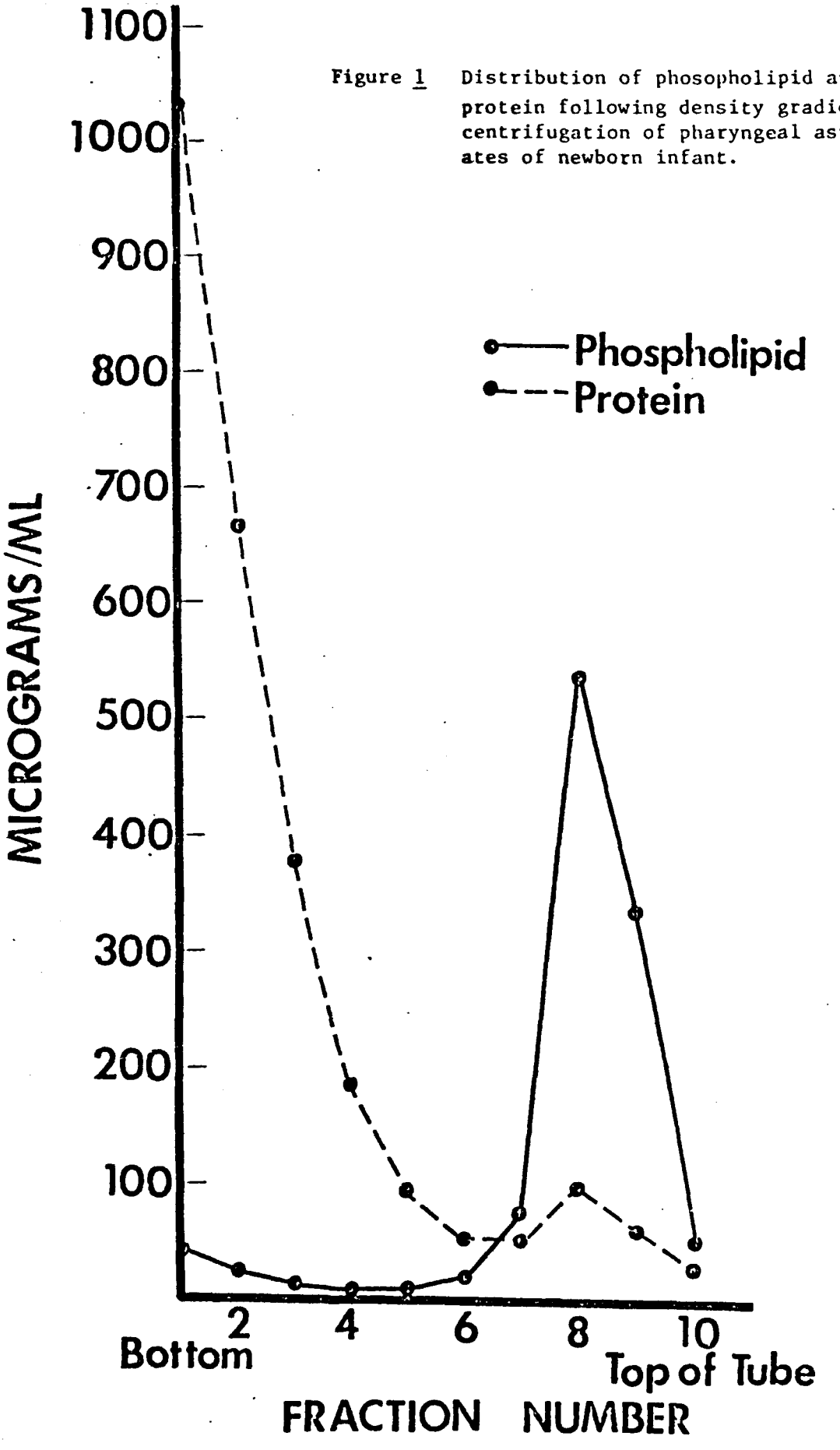

Figure 1 Distribution of phosopholipid and protein following density gradient centrifugation of pharyngeal aspirates of newborn infant.

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13. ABSTRACT

Appended

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Shock Model						
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Shock Treatment						
Steroids in Shock						
Trasylol in Shock						
Epsilon amino caproid acid (EACA) in shock						
Coagulation in Shock						

ABSTRACT

A. Development of a rapid and simple method for lung surfactant purification:

A major problem in the study of human lung surfactant, particularly in pathologic conditions, is the lack of appropriate methods to separate the surfactant from blood components transudated into the air spaces. A rapid and simple method capable of purifying surfactant from alveolar washings, from washings contaminated with plasma and from residual lung tissue (intracellular surfactant) has been developed. The sample, containing 16% NaBr, is placed beneath a two-layer discontinuous gradient of NaBr. After centrifugation, the surfactant is found near the top of the gradient tube at a density of 1.085 at 4°C while the contaminating material remains near the bottom. The chemical composition of surfactant from lung washings of normal animals isolated by this method compares quite favorably with surfactant isolated by much more elaborate and time consuming methods. In addition, a surfactant fraction with a chemical composition very similar to that of the surfactant from normal lung washing can readily be separated from a mixture of plasma and lung washings by this method. Application of the method to the lung tissue remaining after thorough alveolar lavage permits recovery of an intracellular surfactant fraction which has a somewhat different composition than alveolar surfactant isolated from lung washings. This method has also been applied to small samples using as little as 1 ml of starting material for the isolation of surfactant, making the method quite useful for isolating surfactant from human tracheal aspirates in order to monitor clinically surfactant changes in patients with adult respiratory distress syndrome.

B. Biochemical and structural changes of the lung surfactant in endotoxic shock:

(1) Biochemical changes: Purification and biochemical evaluation of the alveolar and intracellular surfactant has already been performed in two pairs of monkeys. One monkey from each pair was infused with 10 mg endotoxin/kg/hr while the other monkey received Ringer's lactate solution only. The animals were killed 10 hours after the onset of endotoxin or saline infusion. Biochemical evaluation of the lung washings from the above monkeys revealed the presence of large excesses of protein in the washing from the endotoxin treated animals due to the pulmonary edema. However, following density gradient centrifugation of the experimental and control washings, contaminating plasma lipids and proteins were eliminated and the lecithin composition of both control and experimental monkeys were similar with the exception that one of the endotoxin injected monkeys (which also revealed prominent pulmonary edema) the percent of the palmitic acid of surfactant lecithin was lower than normal. However, evaluation of the intracellular surfactant failed to demonstrate differences in the biochemical composition of surfactant between control and experimental groups. The above preliminary studies suggest that the early development of "congestive atelectasis" in shock is not associated with apparent profound changes in the surfactant system.

(2) Structural changes: Using the method of total lung fixation (which was found to preserve the surfactant layer and the overall lung architecture) we were able to demonstrate that congestive atelectasis is characterized by multifocal foldings of the alveolar walls which characteristically involve damaged areas of the (injury sensitive) type I epithelium. The multifocal foldings resulted in the formation of small plicated alveoli. The surfactant layer was found to be intact only in non-plicated portions of the alveolar wall and revealed excess focal deposition of myelin figures (surfactant phospho-

lipids) sealing the openings of the folded portions of the alveolar epithelial lining. These findings suggest that the surfactant layer cannot be readily formed at sites of epithelial injury and, therefore, the injured parts of the epithelial lining plicates during expiration.

C. Coagulation and other hematologic changes in endotoxin shock with or without steroid treatment:

Initially we investigated the hematologic changes induced by continuous endotoxin infusion (10/mg/kg/hr) versus bolus endotoxin infusion (10 mg/kg injected over a 10 min. period). A major difference between the two models was the finding that in the bolus model factor XII dropped sharply within 30 min. after endotoxin infusion. By contrast in the continuous infusion model the level of factor XII actually increased after 30 min. but thereafter demonstrated a gradual but progressive drop. All other hematologic and coagulation parameters were essentially the same in both models during the first three hour period after the onset of endotoxoin injection. In the continuous infusion model with or without steroid treatment the following hematologic changes were observed: (1) Leukocytes: marked drop during the first three hours followed by rebound progressive increase thereafter. At 10 hours the leukocyte count had reached the pre-infusion level and at 16-18 hours the animals showed slight leukocytosis. By contrast steroid treated animals showed only mild drop in the leukocyte count at 30 min. Thereafter there was a sharp increase and at 6 hours the leukocyte count ($1000/mm^3$) was 22.5 ± 5.6 (baseline value 10.3 ± 1.8). In addition further progressive increase in the leukocytes count was noted throughout the 24 hour experimental period. (2) Platelets and fibrinogen: they both demonstrated progressive drop in the continuous infusion model, but this drop was significantly less prominent in animals treated with steroids. (3) Coagulation factors: At 30 min. no significant changes were noted. After 3 hours factors V, VIII, and XII demonstrated progressive decrease while the drop of factor VII was marked only at 16-18 hours. Treatment with glucocorticosteroids significantly inhibited the drop of factor VIII but had no statistically significant affect on factor V and XII.

The above hematologic data correlated well with the histochemical and ultrastructural findings which indicated that glucocorticoids inhibit endotoxin induced : (a) sequestration degranulation and fragmentation of the leukocytes in the pulmonary microcirculation, (b) marked endothelial damage in association with interstitial edema, multifocal swelling of the type I epithelium and congestive atelectasis, and (c) early development of diffuse microthrombosis in the sinusoids of the liver (and spleen) as well as centrilobular liver necrosis.

D. The effect of epsilon aminocaproicacid (EACA and trasylol on endotoxin shock:

(1) EACA: Four pairs of monkeys were used. In each pair of monkeys endotoxin was infused continuously at a rate of 10 mg/kg/hr but one monkey from each pair also received EACA 50 or 100 mg/kg/hr. The monkeys were killed in pairs at 6 hours and 16 hours after the onset of endotoxin injection. The results that we have obtained thus far suggest that this drug does not significantly alter endotoxin induced hematologic changes but does appear to inhibit margination

of the leukocytes in the pulmonary vascular bed during the early stages of shock. These changes were associated with significant preservation of the endothelial integrity. In addition, there was evidence that EACA inhibited formation of fibrinous deposits in the microcirculation of the liver and spleen possible because the drug promoted endothelial preservation. In future experiments EACA will be given in smaller doses, 20 mg/kg/hr, because a significant transient hypotensive response was noted rapidly after the onset of EACA administration and this response was more severe with 100 mg EACA/kg/hr. (2) Trasylol: Four pairs of monkeys were infused with endotoxin (10 mg/kg/hr) but one animal from each pair was also given trasylol, 5,000 KIU/kg/hr. The animals were sacrificed 6 or 10 hours after the onset of endotoxin infusion. We have not as yet completed evaluation of the above pilot experiments because we are currently concentrating on the in vitro affects of trasylol on blood coagulation. However, our preliminary findings suggest that the doses employed were large since one animal developed disseminated intravascular thrombosis.